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CAMILA INÉS ZORNOSA TORRES

COMUNICAÇÃO ACÚSTICA EM ANUROS COM ESPECIAL ENFOQUE PARA: EVOLUÇÃO DE PERDA DA VOCALIZAÇÃO DE ANÚNCIO E PRESENÇA DE VOCALIZAÇÃO DE CORTE EM *Bokermannohyla luctuosa*

ACOUSTIC COMMUNICATION IN ANURANS FOCUS ON: EVOLUTION OF THE LOSS OF ADVERTISEMENT VOCALIZATION AND PRESENCE OF COURTSHIP VOCALIZATION IN *Bokermannohyla luctuosa*

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RESUMO

A reprodução é uma das características mais conspícuas da biologia dos anfíbios, tendo uma grande diversidade em estratégias reprodutivas, modos reprodutivos e tipos de comunicação. A comunicação acústica é provavelmente a mais comum nos anuros e dentro desta, a vocalização mais estudada é o canto de anúncio. Os machos geralmente usam essa vocalização para atrair fêmeas para reprodução, as vezes utilizam o canto de corte para estimular e guiar as fêmeas ao sítio de reprodução. Apesar de estas vocalizações serem fundamentais para a maioria de anuros, algumas espécies não emitem o canto de anúncio e, portanto são consideradas mudas. Frequentemente, a ausência deste canto é atribuída ao alto ruído de fundo, sítios permanentes de reprodução, ou a falta de espécies relacionadas no mesmo habitat. Porém, estas sugestões nunca foram testadas num contexto evolutivo. Além disso, o comportamento de corte é pouco estudado nas espécies de anuros tropicais, devido à alta diversidade e à dificuldade de observar dito comportamento no campo. Assim, avaliamos se o ruído do ambiente poderia ser uma pressão seletiva das espécies ou populações de anuros mudos e descrevemos o comportamento de corte e os cantos de anúncio e corte da espécie *Bokermannohyla luctuosa*. Para a primeira parte deste estudo, revisamos as espécies mudas e mapeamos a sua distribuição na filogenia mais completa dos anuros. Estimamos o estado ancestral para o caractere mudo e analisamos a correlação entre o ruído do ambiente e a perda do canto de anúncio de duas famílias (Bufonidae e Hylidae). Em ambas as famílias, a perda do canto é dependente do ruído do ambiente. Assim, o ruído do ambiente provavelmente atua como uma pressão seletiva na perda do canto nos bufonídeos e nos hilídeos. Para a segunda parte relacionada com o comportamento reprodutivo e a comunicação de *B. luctuosa*, observamos a população de Botucatu, gravando as vocalizações com um gravador TASCAM DR680 e filmando os comportamentos usando câmeras filmadoras Sony DCR-SR47 e Sony DCR-TRV460. *Bokermannohyla luctuosa* apresenta um comportamento de corte complexo, que envolve sinais táteis e dois tipos de vocalizações (canto de anúncio e de corte), ambas as vocalizações apresentam estrutura pseudo-pulsada. O canto de corte é semelhante ao de anúncio, porém é mais longo e tem uma frequência dominante menor. A maioria das características do corte da *B. luctuosa* já foram relatadas para outras espécies da mesma família.

ABSTRACT

Reproduction is the most conspicuous characteristic in amphibian biology, with a huge diversity of reproductive strategies, modes and types of communications. Acoustic communication is probably the most common communication in anurans, and, within it, the most studied vocalization is the advertisement call. Males generally use this call to attract females for reproduction, sometimes another call (courtship call) is used to stimulate and guide females to the reproduction site. In spite of these calls being fundamental for most anurans, some species lack the advertisement call and, therefore, are considered mute. Frequently, voicelessness is attributed to high background noise, permanent breeding sites, or lack of closely related species in the same habitat. However, such suggestions have never been tested in an evolutionary context. Also, courtship behavior is poor studied in species from the tropics, due to the high diversity and the difficulty to observe that behavior in the field. Thereby, we evaluated whether habitat noise could be a selective pressure leading to mute species or populations and describe the courtship behavior and the advertisement and courtship call of the species *Bokermannohyla luctuosa*. For the first part, we reviewed the mute species and mapped their distribution onto the most complete anuran phylogeny. We estimated ancestral states of the mute character and tested for correlation between the habitat noise and the voice loss in two families (Hylidae and Bufonidae). In both families, voice loss was dependent of habitat noise as said in a widespread assumption, environmental noise probably acted as a selective pressure on voice loss in bufonids and hylids. For the second part related with the courtship behavior of *B. luctuosa*, we observed the population from Botucatu, recording the vocalizations with a TASCAM DR680 sound recorder and filming the behaviors using a Sony DCR-SR47 and a Sony DCR-TRV460 video camera. *Bokermannohyla luctuosa* presents a complex courtship that involves tactile cues and two types of vocalizations (advertisement and courtship calls), both calls have pseudo-pulsed structure. Courtship call was similar to advertisement call but had a larger duration and a lower dominant frequency. Most of the characteristics of *B. luctuosa* courtship have also been reported for other species of the same family.

SUMÁRIO

Introdução geral

A reprodução dos anfíbios é altamente variável em modos reprodutivos e compreende desde a atração dos pares até a oviposição e o cuidado parental, quando existente (Duellman & Trueb, 1996). Um dos componentes mais importantes na reprodução dos anfíbios é a comunicação. Hailman (1977) estabelece a comunicação como a transferência de informação por meio de um sinal e por um canal entre um emissor e um receptor. Esta é reconhecida pela variação no comportamento do receptor, na presença ou ausência do sinal (Hailman, 1977). Os anfíbios apresentam diferentes tipos de comunicação (acústica, visual, química, sísmica e tátil) em diversas etapas da reprodução, como a atração de parceiros e defesa de sítio de vocalização ou sítio de desova (Duellman & Trueb, 1996; Palermo-Neto & Alves, 2010).

Particularmente, para os anuros a comunicação acústica é a mais difundida e estudada. Essa comunicação está presente na vasta maioria das espécies e é frequentemente utilizada para a atração de parceiros reprodutivos, defesa territorial, defesa contra predadores e competição intrasexual (Wilczynski & Chu, 2001). Já foram reconhecidas diferentes vocalizações em anuros durante a época reprodutiva como: o canto de anúncio, principalmente emitido por machos para atrair a fêmea ou alertar outros machos da ocupação do sítio de canto (Haddad, 1995; Duellman & Trueb, 1996; Wilczynski & Chu, 2001); e o canto de corte, usado como comunicação de curta distância previa ao amplexo, para estimulação e orientação das fêmeas (Toledo *et al.* 2015).

A comunicação tátil em anuros é geralmente utilizada durante o amplexo ou a corte. Já foi relatado o uso destes sinais na identificação sexual, estimulando o amplexo de algumas espécies (*Lithobates sylvaticus*, *Hyla andersonii*, *Ascaphus truei*, *Pipa pipa*) (Duellman & Trueb, 1996). Esses sinais também foram observados envolvendo toques tanto dos machos, quanto das fêmeas durante a corte, como sinal de aceitação do amplexo (Haddad & Sawaya, 2000; Carvalho Jr. *et al*. 2006, Zina & Haddad, 2007; Lima *et al.* 2014). Esse é o caso, por exemplo, de *Aplastodiscus leucopygius*, no qual a fêmea, após de ser atraída pelo macho, toca os membros posteriores dele com as mãos ou com a cabeça, e o macho responde a esse sinal virando na direção dela e tocando-a na cabeça com a região gular ou com o focinho (Haddad *et al.* 2005).

Além de emitirem sinais unimodais, é possível também a comunicação multimodal em anuros, que se refere à produção de mais de um tipo de sinal de maneira simultânea ou

secuencial que pode possuir significado único ou variado (Narins *et al.* 2003; Preininger *et al.* 2009; Grafe *et al.* 2012; Preininger *et al.* 2012; Preininger *et al.* 2013a; Preininger *et al.* 2013b). As vezes, sinais acústicos são emitidos em consonância com visuais, como registrado em *Allobates femoralis, Staurois parvus*, *Sataurois latopalmatus, Engystomops pustulosus* (Taylor *et al.* 2008; Preininger *et al.* 2009; Grafe *et al.* 2012; Preininger *et al.* 2013a).

A ausência de vocalizações em anuros é rara e pode ser atribuída à presença de ruído de fundo intenso ou uso de locais permanentes de reprodução (Emerson & Igner, 1992). Neste caso, formas alternativas de comunicação podem ser empregadas para as espécies se comunicarem. Este é, por exemplo, o caso de *Limnonectes blythii* que se comunica visualmente para atrair a fêmea (Emerson, 1992) e *Leiopelma hamiltoni* que se comunica quimicamente para defender seu território reprodutivo (Lee & Waldman, 2002; Waldman & Bishop, 2004). No entanto, ainda não sabemos como muitas das espécies mudas se comunicam, nem se há alguma relação entre todas elas (seja ecológica ou filogenética). Neste sentido, o presente estudo realizou uma revisão bibliográfica das espécies mudas, ligando a filogenia e ecologia destas espécies e testando umas das hipóteses que explica a ausência do canto de anúncio em anuros. Além disso, realizamos a descrição do comportamento de corte e as vocalizações de anúncio e corte da espécie *Bokermannohyla luctuosa* da população de Botucatu.

<u>CAPÍTULO 1</u>: "DOES ENVIRONMENTAL NOISE DRIVE THE LOSS OF ADVERTISEMENT CALL IN ANURANS?"

DOES ENVIRONMENTAL ABIOTIC NOISE DRIVE THE LOSS OF ADVERTISEMENT CALL IN ANURANS?

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Abstract

Acoustic communication is probably the most common communication in anurans, and, within it, the most studied vocalization is the advertisement call. Mainly males use this call to attract conspecific females for reproduction. In spite of this call being fundamental for most anurans, some species lack the advertisement call and, therefore, are considered mute. Frequently, voicelessness is attributed to high background noise, permanent breeding sites, or lack of closely related species in the same habitat. However, such suggestions have never been tested in an evolutionary context. We evaluated whether habitat noise could be a selective pressure leading to mute species or populations. We reviewed the mute species and mapped their distribution onto the most complete anuran phylogeny available to date. We estimated ancestral states of the mute character. We listed 40 mute species belonging to 7 families, and suggested that the mute trait is the ancestral state in the phylogeny we use. We further investigated two families, Hylidae and Bufonidae, which have the highest number of mute species represented in the phylogenetic tree, and tested the relation between voice loss and ambient noise level. The rate of evolution of the advertisement call was dependent of habitat noise, as a widespread assumption, environmental noise probably acts as a selective pressure on voice loss in bufonids and hylids. Based on our results, we indicate the need of additional natural history studies, focusing on alternative communication modes among mute species in order to provide new insights about possible selective forces that are taking place in this case.

Keywords: Amphibian communication, ancestral reconstruction, Pagel´s correlation, phylogeny, voiceless frogs evolution, background noise.

INTRODUCTION

Animal communication is central in evolutionary biology and of fundamental importance in species natural history, as it mediates reproductive, defense, territorial, aggressive, and social interactions (Noble 1998; Narins 2001; Fitch 2006; Toledo and Haddad 2009; Toledo et al. 2011). Communication signals are under constant selection for maximizing the information transmitted, providing unambiguous cues and adapting to various

habits in form of different modes of communications, all this aspects let it remain in the evolutionary history of the species (Hauser 1996).

In anurans there are at least five channels of communication: acoustic, visual, chemical, tactile, and seismic, in which acoustic is by far the most common (Vitt and Caldwell 2009). This communication mode is present at different phases of the reproduction, such as in mate attraction and defense of calling or spawning sites (Wilczynski and Chu 2001; Palermo-Neto and Alves 2010). Consequently, the acoustic channel has been recognized as the most important communication mode in anurans (Wells 2007; Dorcas et al. 2010). Vocalizations have a tremendous importance in anuran communication because they transmit relevant information, such as individual's location, size, reproductive status, aggressive motivation, or can mediate predator-prey interactions (Davis 1987; Bee and Gerhardt 2001; Ryan 2001; Simmons 2004; Wells 2007; Toledo and Haddad 2009; Toledo et al. 2015).

Fourteen different types of calls, subdivided into three main categories, have been described for anurans (Toledo et al. 2015; Köhler et al. submitted). The most common vocalization in anurans is the advertisement call, produced principally by males in the reproductive season, but it can also be produced by females (Duellman and Trueb 1994; Haddad 1995; Wilczynski and Chu 2001; Toledo et al. 2015; Preininger et al. in press). It is highly variable and can be a simple note or a series of identical notes, it can be produce in the frequency of audible sound, infrasound or ultrasound (Toledo et al. 2015). Advertisement calls can also combine those frequencies, as registered for the call of *Odorrana tormota*, which combines ultrasound and audible frequencies (Feng et al. 2006). Males produce advertisement calls to attract conspecific females or males to join the chorus; it allows them to distribute themselves within a calling site and for the female to localize them individually while it also transmits information about male size that has an important role in sexual selection and in physical combats (Toledo et al. 2015).

Given the importance of advertisement calls, it is surprising to find that some species lack such vocalizations, referred to as mute or voiceless frogs (Emerson and Inger 1992; Haddad and Giaretta 1999; Rödel et al. 2003; Wells 2007; Toledo et al. 2015). Mute species are expected to use alternative channels of communication in place of advertisement calls (Emerson 1992; Lee and Waldman 2002; Waldman and Bishop 2004). This prediction was corroborated in the case of males of *Limnonectes blythii* (Dicroglossidae), which uses visual signaling to attract females (Emerson 1992), and males of *Leiopelma hamiltoni* (Leiopelmatidae), which communicates chemically to defend its reproductive territory (Lee and Waldman 2002; Waldman and Bishop 2004).

The absence of advertisement calls has been attributed to the presence of intense background noise, the use of permanent breeding sites, or the presence of closely related species in the same habitat (Emerson and Inger 1992). Presence of low frequency background noise could mask anurans vocalizations, which would need to use more energy to being heard. However, the hypotheses linking voicelessness and breeding environment, to our knowledge, has not been tested yet. In this study, we investigated whether species living in noisy environments have a higher propensity to lack advertisement call. To test this hypothesis we did a correlation between both characters and searched for the ancestral character state of the advertisement call.

METHODS

Data acquisition and character mapping

We defined as mute or voiceless, anurans that do not emit advertisement calls; the presence of other call types was not considered. We searched for mute anuran species in the available literature, searching the online databases and university libraries using the keywords: voiceless frog, mute frog, reproductive biology, communication, reproduction, Amphibia, ecology, and specific epithets of species suggested to be mute. Additionally, some information was gathered from personal communications. Information about habits and habitat of each species were collected from the IUCN redlist website (IUCN 2015). Taxonomy follows Frost (2016).

The presence of advertisement call was coded as a binary character (0: absent, 1: present) for each species, species having mute and calling populations were consider as calling species. After compilation of the data, we mapped advertisement call absence and their abundance on the most recent and comprehensive global amphibian phylogeny (Pyron 2014). This phylogeny includes representatives of more than 90 % of the currently recognized genera and over 3,300 amphibian species (Pyron 2014), we considered mute species, and those not mute belonging to the Bufonidae and Hylidae. To prune the species we used the function *prune.sample* of the package "picante" from the R platform (Kembel et al. 2014).

As some reports of absence of advertisement calls could result from a lack of natural history knowledge, we considered mute only those species that have been reported to be voiceless in more than one reference, or those that have been reported voiceless for a long time (more than five years). If these were not the case (e.g., just one recent reference indicates

that the species is mute), we considered the species as probably mute. We also categorized the families in ranges (0%, 0.1-5%, 5.1-50%, >50%) considering the number of mute species and the total number of species of the family.

Environmental noise was coded as binary (0: not noisy and 1: noisy). We considered noisy habitats those with waterfalls and rapid streams; all other cases (such as terrestrial, lentic environments, and slow rivulets) were considered not noisy habitats.

Ancestral Character Estimation

Based on the phylogenetic hypothesis published by Pyron (2014), we estimated ancestral presence of advertisement call in two different ways: one in separate phylogenies for bufonids and hylids, without considering mute species of other families; and the other in a unique phylogeny that considers bufonids, hylids, and mute species of these and other five families. We used the function *ace* from the R package "ape", with the symmetrical model (SYM) (Paradis et al. 2015). The ancestral states reconstruction of discrete characters considered the maximum likelihood estimation, using the probabilities of each character states at each node of the tree, assuming that all states of the character are possible at each node (even if some are more likely or more parsimonious than others) and that each character evolves at a constant rate over the tree (Pagel 1994; Cunningham et al. 1998). The probabilities were determined by the distribution of the character state in the tip species, by the rate of character evolution and by the inter-nodal branch lengths (Cunningham et al. 1998).

Influence of abiotic environmental noise on voicelessness

In order to test whether the loss of advertisement call was related to the environmental noise, we did a Pagel´s correlation test (Pagel 1994), using information of families Bufonidae and Hylidae, and complete information of all the mute species. We used a total of 495 species pruned from the phylogeny published by Pyron (2014). The Pagel´s correlation test uses a continuous-time Markov model to characterize evolutionary changes along each of the branches of a phylogeny (Pagel 1994). This method tests the hypothesis of correlation between two binary characters by comparing the fit of two models to the observed data set.

The first model treats the two characters as evolving independently, the goodness of fit of this model is then compared to a more complex model in which the characters evolve in a correlated fashion (dependent model) (Pagel 1994). The dependent model is justified as a representation of the data if it fits the data significantly better than the independent model; their significance is assessed by means of a likelihood ratio statistic (Pagel 1994).

Thereby, we built an independent and dependent model of correlation and compared their goodness of fit to our data. We selected the best fitting model using the Akaike's Information Criterion (AIC), and the log-likelihood statistic. This analysis was implemented in the R platform using the "phytools" package and the function *fitPagel*, we also add the argument *dep.var = "x"* to fit the direction of our hypothesis (advertisement call rate of evolution depends on the environmental noise rate of evolution) (Pagel 1994; Revell 2012).

RESULTS

We listed 40 mute anuran species, assigned to 7 families, dispersed over the order Anura (Fig. 1) and globally distributed (Table 1). Leiopelmatidae is the only family with all species mute; three families have between 5.1 and 50 % of mute species, and three of the seven families have from 0.1 to 5 % of mute species. A clade with three sister genera containing mute species (*Incilius*, *Anaxyrus*, and *Rhinella*) was highlighted in the family Bufonidae (Fig. 2). Several mute species were recognized in the family Hylidae, including a genus, *Megastomatohyla*, containing only mute species (Fig. 3). Families Bufonidae and Hylidae were selected to more detailed analysis because of the high number of mute species and its representativity in the tree.

The advertisement call loss occurred four times within the Bufonidae and three times within the Hylidae, when using different phylogenies (Fig. 4), being the presence of advertisement call the ancestral state. When using the unique phylogeny of both families and including mute species of other families, the ancestral character state was the absence of the advertisement call (Fig. 5), after that the advertisement call loss eleven times within the phylogeny. The Pagel's correlation test showed that both models are different (likelihood-ratio = 4.04; $P = 0.04$). The best fitting model for our data set was the one that shows that the rate of evolution of the advertisement call depends on the rate of evolution of the environmental noise (Independent model: AIC = 544.09, Log-likelihood = -270.05; Dependent model: AIC = 542.05, Log-likelihood = -268.03).

DISCUSSION

For the first time we provided, based on a comprehensive analysis, support to the previous hypothesis that the lack of advertisement calls is related to the presence of abiotic background noise (Emerson and Inger 1992; Wells 2007). Responses to environmental noise as a selective pressure varies among anuran clades, as some lineages or clades evolved to use alternative signals or voicelessness (Rödel et al. 2003; Grafe et al. 2012; Stratenberger et al. 2013). For example, *Leiopelma* species use visual and chemical communication (Green 1988; Lee and Waldman 2002; Waldman and Bishop 2004; Bell 2010) and *Acanthixalus* spp. produce volatile substances as an alternative communication (Rödel et al. 2003; Stranberger et al. 2013). Therefore, anurans showed to be variable in relation to the different outcomes selected under the influence of background noise.

Furthermore, some studies suggest that environmental characteristics can influence the evolution of long-range acoustic signals and in some cases can even modulate species vocalizations (Bosch and De la Riva 2004). Different vocal characteristics are linked with environmental noise. For example, some species that reproduce in streams have advertisement calls with high dominant frequency, suggesting that advertisement calls have evolved towards higher frequencies, thereby avoiding spectral overlap with background noise (Goutte et al. 2013; Röhr et al. 2015). These frogs may also use visual signaling and in some cases ear tuning acting as a frequency band filter (Goutte et al. 2013; Röhr et al. 2015; de Sá et al. 2016).

The absence of advertisement call in frogs has been considered as an scarce evolutionary event (Wells 2007), as few species were reported as mute. Nonetheless, we showed that even the number of mute species is low in proportion to the amphibian species of the world (Frost 2016), it is considerably high considering that acoustic communication is the principal communication mode in anurans (Wells 2007; Toledo et al. 2015). It is possible that we over-estimated the number of mute species, as a consequence of insufficient natural history data. On the other hand, we could be also under-estimating this number, when assuming that several species produce advertisement calls (when there is no studies that suggest the contrary). We suggest that voiceless ancestral condition can varies depending on the method used, and we propose that voiceless is an ancestral condition, as Leiopelmatidae (a family sister to all other families in the Anura clade) (Frost et al. 2006; Pyron and Wiens 2011; Pyron 2014), Gymnophiona and Caudata (other Orders of Amphibia, being caecilinas

sister to Anura: Fig. 1), lacks advertisement call (Wells 2007), agreeing to what was previously proposed (Stephenson and Stephenson 1957).

Otherwise, the loss of advertisement call in anurans evolved many times independently, as also observed for Bornean voiceless frogs that lost the advertisement call twice (Emerson and Berrigan 1993). Such high frequency of losses may imply that the advertisement call, although a very important type of communication in anurans (Wells 2007; Toledo et al. 2015), could be easily lost during evolutionary history. An intermediate state could be the lost of the advertisement call primarily in the reproductive communication, being overcome by a different communication modality. For example, visual communication plays a major role in some cases, such as in the genera *Brachycephalus* (Pombal Jr. et al. 1994; Araújo et al. 2012).

At last, additional studies focused on the reproductive biology and communication of voiceless species are necessary to assure their proper classification as mute or not. Advertisement calls and calling behavior are highly variable and species may be misclassified as mute if they produce very rare or low intensity calls (see Blair and Pettus 1954; Wells 2007; Wells 1977; Toledo et al. 2015). Some species have been incorrectly reported as mute for a long time, such as *Incilius alvarius* (Bufonidae), *Mantidactylus guttulatus* (Mantellidae), and *Insuetophrynus acarpicus* (Rhinodermatidae) (Blair and Pettus 1954; Diaz et al. 1983; Sullivan and Malmos 1994; Vences et al. 2004). Others have mute and calling populations as *Bufo bufo* (Bufonidae), *Anaxyrus boreas* (Bufonidae), *Limnonectes kuhlii* (Dicroglossidae), and *Limnonectes blythii* (Dicroglossidae) (Black and Brunson 1971; Höglund and Robertson 1988; Emerson 1992; Matsui 1995; Orlov 1997; Emerson and Ward 1998; Tsuji and Lue 1998; Wells 2007). These last cases should be target of subsequent studies, as they may provide comparative scenario (between mute and vocal populations), helping to understand better loss of call evolution. Besides this, it is noteworthy that some mute species can also emit other types of calls, such as release and distress calls (e.g., Schuierer 1962; Black and Brunson 1971; Bell 1978; Penna and Veloso 1987; Green 1988; Höglund and Robertson 1988; Jacobson and Vanderberg 1991; Toledo and Haddad 2009; Grant and Bolívar-G 2014). Therefore, the terminology mute should be avoided or clearly defined when necessary. We also suggest studies that involve anatomical evaluation of mute species.

ACKNOWLEDGMENTS

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Table 1. The 40 mute or probably mute (indicated by an asterisk) anuran species of the world, countries where they naturally occur, breeding habitat, and references indicating that they are mute.

Species	Distribution	Breeding habitat	Reference
Alsodidae			
*Alsodes montanus**	Chile	Streams	Penna and Veloso 1987
*Alsodes tumultuosus**	Chile	Streams	Penna et al. 1983
*Alsodes valdiviensis**	Chile	Streams	Formas et al. 2002
Bufonidae			
*Incilius periglenes**	Costa Rica	Pools	Jacobson and Vanderberg 1991
Incilius holdridgei	Costa Rica	Streams	Novak and Robinson 1975
*Incilius peripatetes**	Panama	Unknown	Novak and Robinson 1975
*Incilius epioticus**	Costa Rica and Panama	Unknown	Novak and Robinson 1975
Incilius fastidiosus	Costa Rica and Panama	Streams	Novak and Robinson 1975
Anaxyrus exsul	United States of America	Pools and Streams	Schuierer 1962
Anaxyrus nelsoni	United States of America	Pools	Wells 2007
*Rhinella paraguas**	Colombia	Unknown	Grant and Bolivar 2014
*Rhinella acrolopha**	Colombia	Unknown	Novak and Robinson 1975
*Rhinella spinulosa**	Argentina, Chile, Bolivia and Peru	Lakes, streams and pools	Penna and Veloso 1981
Hylidae			
Bokermannohyla izecksohni	Brazil	Streams	Jim and Caramaschi 1979
Plectrohyla siopela	Mexico	Streams	Wells 2007
Plectrohyla	Mexico	Streams	Canesco-Márquez et al.

ameibothalame			2002
Charadrahyla altipotens	Mexico	Streams	Duellman 1970
Megastomatohyla mixomaculata	Mexico	Streams	Wells 2007
Megastomatohyla pellita	Mexico	Streams	Wells 2007
Megastomatohyla mixe	Mexico	Streams	Wells 2007
Megastomatohyla nubicola	Mexico	Streams	Wells 2007
Hylodidae			
Hylodes vanzolinii	Brazil	Streams	L. F. Toledo (unpubl. data)
Megaelosia apuana	Brazil	Streams	Pombal et al. 2003
Megaelosia bocainensis	Brazil	Streams	Giaretta et al. 1993
Megaelosia boticariana	Brazil	Streams	Giaretta & Aguiar, 1998
Megaelosia goeldii	Brazil	Streams	Giaretta et al. 1993
Megaelosia jordanensis	Brazil	Streams	Heyer, 1983
Megaelosia lutzae	Brazil	Streams	Giaretta et al. 1993
Megaelosia massarti	Brazil	Stream	Giaretta et al. 1993
Hyperoliidae			
Acanthixalus sonjae	Ghana	Tree cavities	Rödel et al. 2003 Stranberger et al. 2013
Acanthixalus spinosus	Cameroon, Congo, Nigeria, Gabon, Democratic Republic of the Congo	Tree cavities	Rödel et al. 2003 Stranberger et al. 2013
Leiopelmatidae			

Leiopelma archeyi	New Zealand	Shallow depressions beneath logs	Bell 2010
Leiopelma hamiltoni	New Zealand	Unknown	Bell 2010
Leiopelma hochstetteri	New Zealand	Land, shallow water	Bell 2010
Leiopelma pakeka	New Zealand	Moist depressions under logs, rocks or vegetation	Bell 2010
Telmatobiidae			
*Telmatobius halli**	Chile	Unknown	Penna and Veloso 1987
*Telmatobius marmoratus**	Peru, Bolivia, Chile, Argentina	Streams, waterfalls	Penna and Veloso 1987
Telmatobius pefauri	Chile	Streams	Penna and Veloso 1987
*Telmatobius peruvianus**	Chile and Peru	Streams	Penna and Veloso 1987
*Telmatobius zapahuirensis**	Chile	Streams	Penna and Veloso 1987

FIGURES

Fig. 1 Distribution of mute anuran families (colored branches) in the anuran phylogeny, with caecilians and salamanders as outgroups (based on Pyron 2014). In parenthesis number of mute species and total numer of species of each family.

Fig. 2 Distribution of mute character (colored branches) in Bufonidae. In parenthesis number of mute species and total numer of species of each genus.

Fig. 3 Distribution of mute character (colored branches) in Hylidae. In parenthesis number of mute species and total numer of species of each genus.

Fig. 4 Ancestral character estimation of advertisement call presence in bufonids (A) and (B) hylids in separate phylogenies without mute species of other families. Red arrows indicate branches were the mute character appears in the phylogeny.

Fig. 5 Ancestral character estimation of advertisement call presence in bufonids, hylids and mute species belonging to other families in a unique phylogeny. Black portions in the pie plot represent the probability of lacks advertisement call. Red portions in the pie plot represents the probability of have advertisement call.

Fig. 1

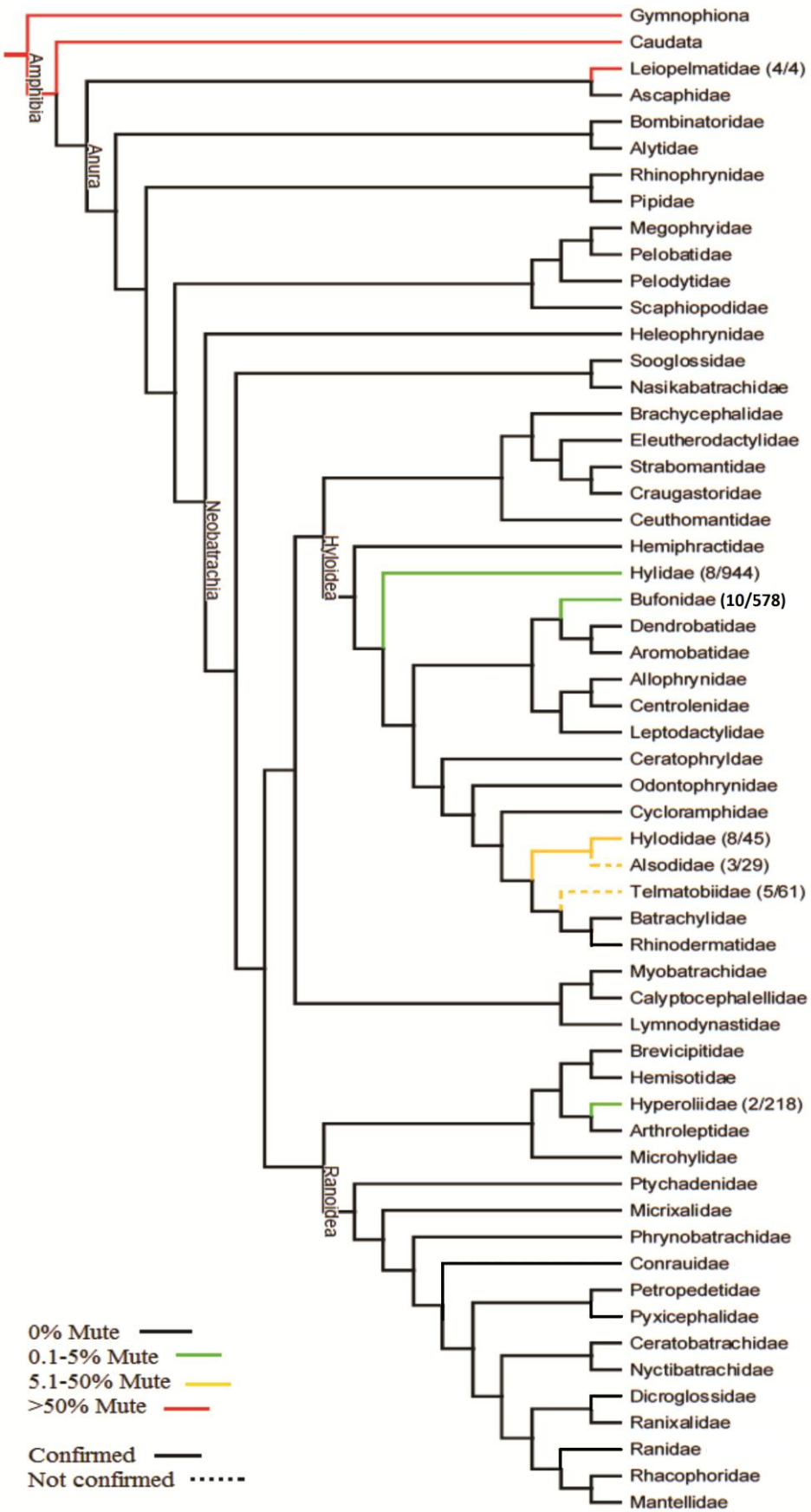

Amphibia
Anura
Neobatrachia
Hyloidea
Ranoidea
Gymnophiona
Caudata
Leiopelmatidae (4/4)
Ascaphidae
Bombinatoridae
Alytidae
Rhinophrynidae
Pipidae
Megophryidae
Pelobatidae
Pelodytidae
Scaphiopodidae
Heleophrynidae
Sooglossidae
Nasikabatrachidae
Brachycephalidae
Eleutherodactylidae
Strabomantidae
Craugastoridae
Ceuthomantidae
Hemiphractidae
Hylidae (8/944)
Bufonidae (10/578)
Dendrobatidae
Aromobatidae
Allophrynidae
Centrolenidae
Leptodactylidae
Ceratophryidae
Odontophrynidae
Cycloramphidae
Hylodidae (8/45)
Alsodidae (3/29)
Telmatobiidae (5/61)
Batrachylidae
Rhinodermatidae
Myobatrachidae
Calyptocephalellidae
Lymnodynastidae
Brevicipitidae
Hemisotidae
Hyperoliidae (2/218)
Arthroleptidae
Microhylidae
Ptychadenidae
Micrixalidae
Phrynobatrachidae
Conrauidae
Petropedetidae
Pyxicephalidae
Ceratobatrachidae
Nyctibatrachidae
Dicroglossidae
Ranixalidae
Ranidae
Rhacophoridae
Mantellidae
0% Mute
0.1-5% Mute
5.1-50% Mute
>50% Mute
Confirmed
Not confirmed

Fig. 2

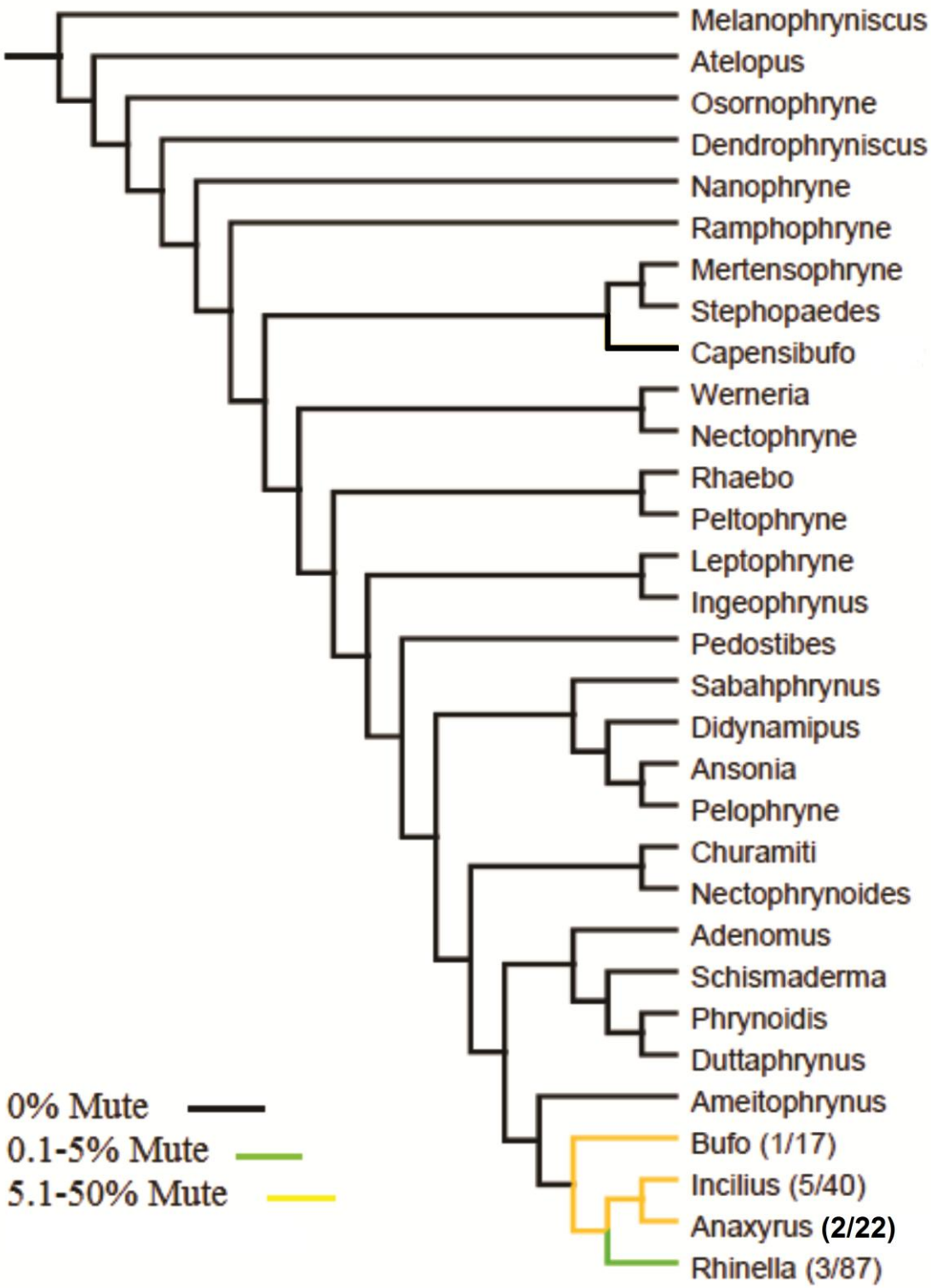

Melanophryniscus
Atelopus
Osornophryne
Dendrophryniscus
Nanophryne
Ramphophryne
Mertensophryne
Stephopaedes
Capensibufo
Werneria
Nectophryne
Rhaebo
Peltophryne
Leptophryne
Ingeophrynus
Pedostibes
Sabahphrynus
Didynamipus
Ansonia
Pelophryne
Churamiti
Nectophrynoides
Adenomus
Schismaderma
Phrynoidis
Duttaphrynus
Ameitophrynus
Bufo (1/17)
Incilius (5/40)
Anaxyrus (2/22)
Rhinella (3/87)
0% Mute
0.1-5% Mute
5.1-50% Mute

Fig. 3

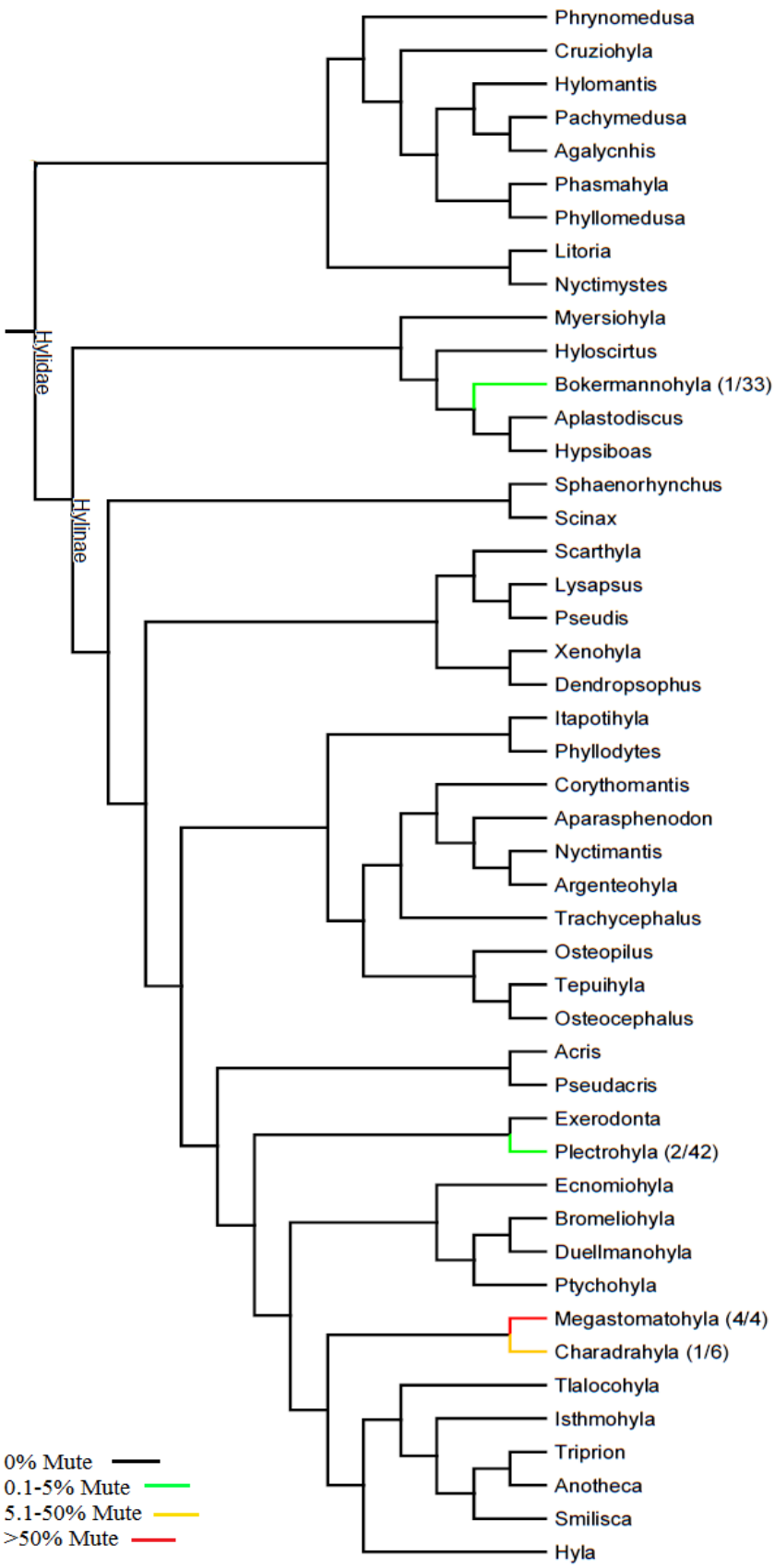

Phrynomedusa
Cruziohyla
Hylomantis
Pachymedusa
Agalycnhis
Phasmahyla
Phyllomedusa
Litoria
Nyctimystes
Myersiohyla
Hyloscirtus
Bokermannohyla (1/33)
Aplastodiscus
Hypsiboas
Sphaenorhynchus
Scinax
Scarthyla
Lysapsus
Pseudis
Xenohyla
Dendropsophus
Itapotihyla
Phyllodytes
Corythomantis
Aparasphenodon
Nyctimantis
Argenteohyla
Trachycephalus
Osteopilus
Tepuihyla
Osteocephalus
Acris
Pseudacris
Exerodonta
Plectrohyla (2/42)
Ecnomiohyla
Bromeliohyla
Duellmanohyla
Ptychohyla
Megastomatohyla (4/4)
Charadrahyla (1/6)
Tlalocohyla
Isthmohyla
Triprion
Anotheca
Smilisca
Hyla
Hylidae
Hylinae
0% Mute
0.1-5% Mute
5.1-50% Mute
>50% Mute

Fig. 4

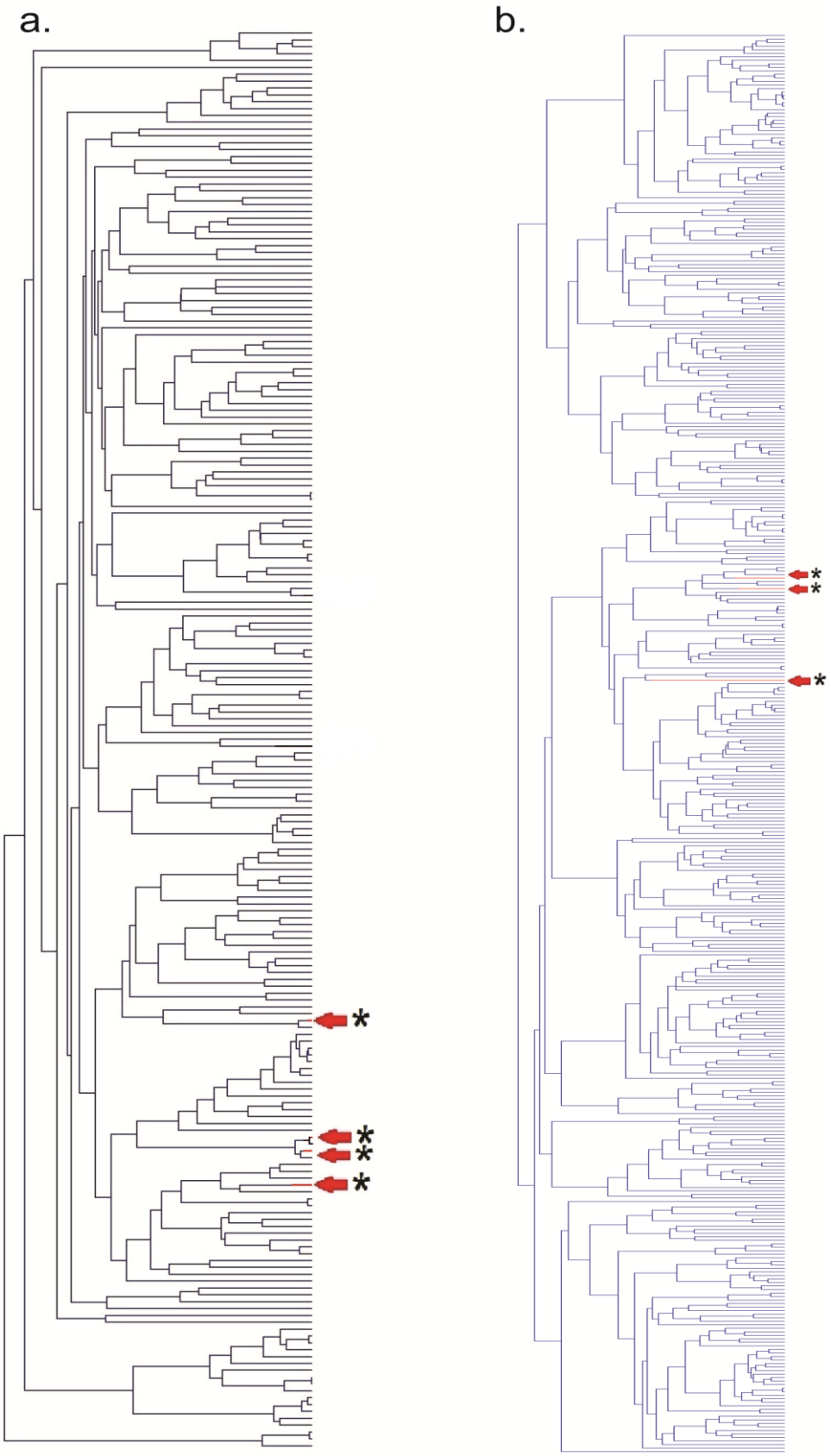
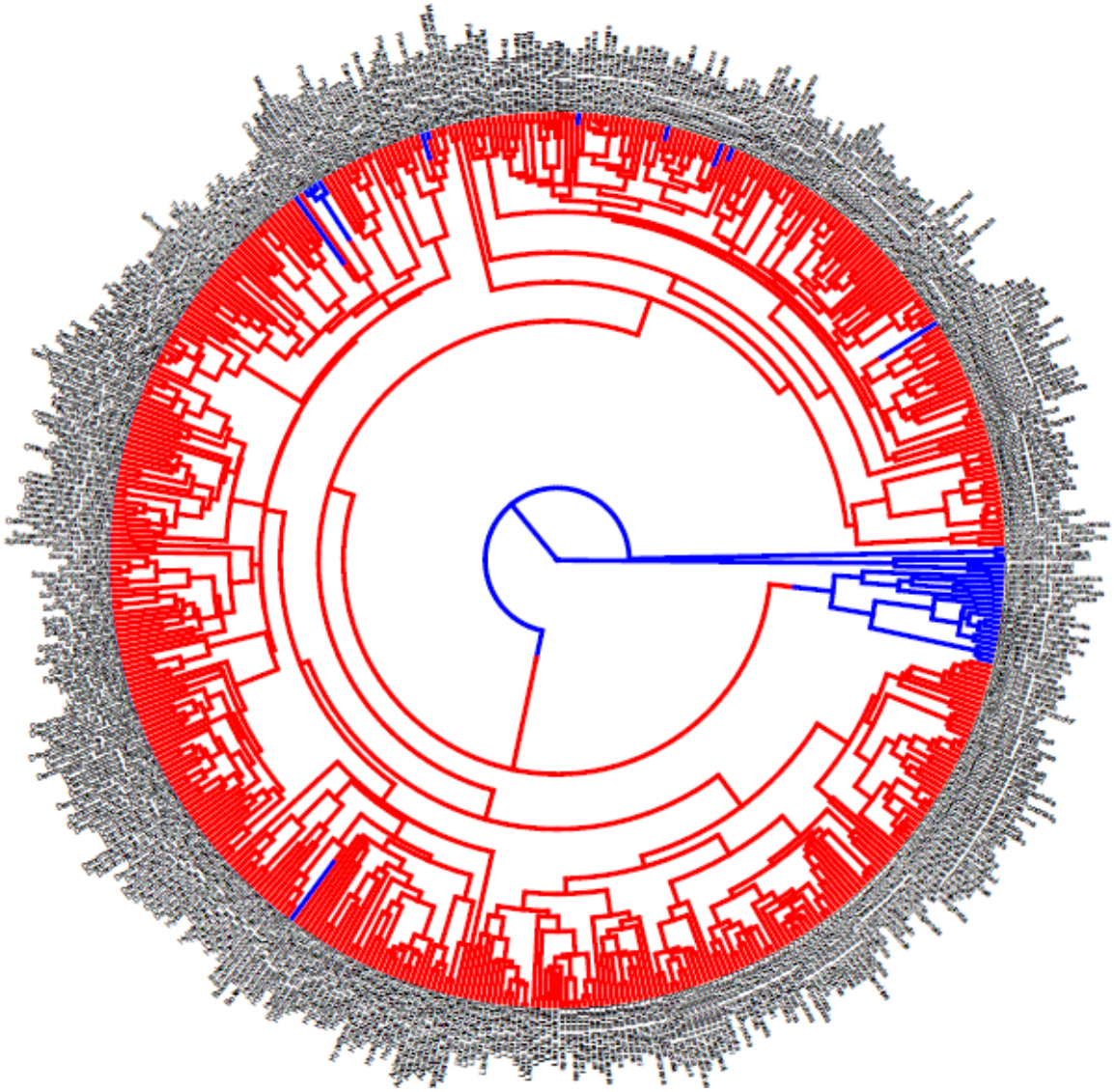


Fig. 5



<u>CAPÍTULO 2</u>: "COURTSHIP BEHAVIOUR OF *Bokermannohyla luctuosa* (HYLIDAE, COPHOMANTINAE) AND GEOGRAPHIC VARIATION OF ADVERTISEMENT CALL"

COURTSHIP BEHAVIOUR OF *Bokermannohyla luctuosa* (HYLIDAE, COPHOMANTINAE) AND GEOGRAPHIC VARIATION OF ADVERTISEMENT CALL

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Abstract

Reproduction is the most conspicuous characteristic in amphibian biology, because there is a great diversity of reproductive modes. In order to reproduce, male anurans generally display acoustic communication and complex courtship behaviors. Hylids have one of the most variable reproductive repertoires, however we lack information for many species, especially in regard to courtship and acoustic communication. Therefore, we studied *Bokermannohyla luctuosa*, a species with the reproductive behavior poorly documented. Hence, we described for the first time its complex courtship, involving tactile communication (touches between the pair) and two types of vocalizations: advertisement and courtship calls. These calls presented pseudo-pulsed structure. The courtship call was similar to advertisement call but had a larger duration, lower dominant frequency and lower relative peak power. Most of the characteristics of *B. luctuosa* courtship have also been reported for other species of the subfamily Cophomantinae, such as *Bokermannohyla nanuzae, Aplastodiscus leucopygius, A. arildae, A. perviridis*, and *Hypsiboas rosenbergi.*

Key words: Advertisement call, axillary amplexus, reproduction, breeding.

INTRODUCTION

Courtship is fundamental for many amphibian species, contribute their reproductive fitness (Duellman & Trueb, 1996). During courtship, amphibians use different types of signals (acoustic, visual, tactile and chemical) to attract mates. Among these, the most commonly reported are chemical (mainly in salamanders), acoustic and tactile (mainly in anurans) (Wells, 1977). Besides this, secondary structures are also important in some species, as enlargement of the cloacal glands in male salamanders or presence of nuptial excrescences in male anurans (Duellman & Trueb, 1996).

However, courtship is just one aspect of reproduction. Traditionally anuran reproductive strategies are categorized into 39 reproductive modes, that includes simple modes as eggs laid in ponds where tadpoles will develop, and more complex modes with eggs swallowed by the mother, completing the subsequent development in its stomach (Haddad & Prado, 2005). Additionally, eggs are deposited in different types of nests, besides gelatinous egg masses, such as foam or bubble nests, on the water, ground or above trees (Haddad & Prado, 2005). Furthermore, anurans vary in their development, from indirect (with tadpoles or larval stage), to direct as in the terrestrial eggs of brachycephaloids (Haddad & Prado, 2005; Wells, 2007; Haddad *et al.* 2013). In spite of such high diversity of breeding modes, many, if not most of the anurans, and especially in the Tropical region, lack information about their reproductive biology. Studies are even scarcer if the considered aspect is courtship. Therefore, such known diversity may be underestimated, highlighting the need of studying reproductive behavior in natural conditions.

Among anurans, the genus *Bokermannohyla* (Hylidae) is interesting to conduct studies of reproductive biology. As breeding biology is still poorly studied for the genus *Bokermannohyla*, the information about their courtship is even more scarce in the literature, this information is known only for 3 of the 32 species of the genus: *B. ibitiguara, B. nanuzae,* and *B. alvarengai* (Nali & Prado, 2012; Lima *et al.* 2014; Centeno *et al.* 2015). Species of the *Bokermannohyla circumdata* group breeds in streams, where males generally emit advertisement calls perched on the marginal vegetation or on the ground near the stream (Caramaschi & Feio, 1990; Carvalho *et al.* 2012). Courtship description is only available for *B. nanuzae,* a species that uses visual (face wiping), tactile (alternating touches in different parts of the body), and acoustic signaling (Lima *et al.* 2014).

Composition of the advertisement call in this group varies from 1 to 30 notes, lasting for up to 7.6 seconds (Napoli & Caramaschi, 2004; Carvalho *et al.* 2012; Gaiga *et al.* 2013; Lima *et al*. 2014). Courtship calls, present in at least *B. nanuzae*, differ from the advertisement calls by having higher rate of emission (from 21 to 44 notes/minute) and longer note duration (from 0.34 to 0.61 s) (Lima *et al.* 2014). Some congeneric species are commonly found in bromeliads (Napoli & Pimenta, 2003; Napoli & Caramaschi, 2004; Napoli & Juncá, 2006; Napoli & Pimenta, 2009; Carvalho *et al.* 2012). However, in most of the cases these females lay their eggs in nearby streams or ponds (Carvalho *et al*. 2012; Haddad *et al.* 2013). Besides that, data available on the reproduction of *Bokermannohyla* spp. focus on its acoustical communication during the breeding season (Carvalho *et al*. 2012; Gaiga *et al.* 2013).

For *B. luctuosa* the advertisement call has been described as composed (with two notes), with frequencies varying from 0.3 to 2.0 kHz, emitted from the ground or perched on vegetation (Pombal & Haddad, 1993; Carvalho *et al*. 2012; Gaiga *et al.* 2013). Call descriptions are available for populations of Serra do Japi (Jundiaí) and Atibaia, both localities in the state of São Paulo, Brazil. However, no information is available about its courtship behavior. Therefore, based on field observations in Botucatu (São Paulo, Brazil) we describe its courtship, including egg laying site and male-female acoustic communications before and during the amplexus. Additionally we compare the advertisement call of these three populations (Jundiaí, Atibaia and Botucatu), as a taxonomic confirmation method, because *B. luctuosa* has not been reported to Botucatu.

METHODS

We conducted field observations at “Sítio Santo Antônio da Cascatinha” located in the district of Rubião Junior, municipality of Botucatu, state of São Paulo, southeastern Brazil (22°53’42.9” S, 48°29’28” W). The study site is included in the Atlantic forest biome, has an elevation of 850 meters, and is consider a semideciduous forest fragment with native plants surrounded by an agriculture matrix (Figure 1A). Inside this fragment there is a stream with approximately 5 to 60 cm of depth, 30 to 100 cm wide, with crystalline water and sandy bottom (Figures 1B-C).

We studied the population of *Bokermannohyla luctuosa* of this fragment between November 2014 and March 2015, during its reproductive season (Pombal Jr. & Haddad, 1993) totaling 37 nights. Specimens were found by *Ad libitum* active search followed by focal animal observations from 19:00 to 3:00 (Altmann, 1974). We filmed the specimens using a Sony DCR−SR47 and a Sony DCR−TRV460 video camera, using a red light lantern or the night vision mode of the cameras. We recorded the vocalizations with a TASCAM DR680 sound recorder coupled to a Sennheiser unidirectional microphone, at a sample frequency of 48 kHz and a sample size of 24 bits.

Prior to analyses, frequencies over 3 kHz were filtered out. Calls were normalized (removing DC offset, centering on 0.0 vertically, and to the maximum amplitude of –1.0 dB) in Audacity 2.1.0. Treated files were saved as 16 bits WAV files. We then analyzed these recordings in Raven Pro 1.4 (Charif *et al.* 2010). The configurations adopted were: 70% brightness, 80% contrast, and a Fast Fourier Transform length (FFT) of 1200.

To describe the calls we used the term pseudo-pulses, here defined as a very short sound with a low frequency resolution and imperfect pulsation (Robilliard *et al*. 2006; Araújo, 2011; see also Fig. 3B). Spectral parameters were measured in the spectrogram and temporal parameters were measured in the oscillogram.

The peak of dominant frequency was obtained with the Peak Frequency function. We used the Frequency 5% and Frequency 95% functions to obtain the minimum and maximum frequencies respectively. The Frequency 5% function corresponds to the minimum frequency cutting the 5% of the energy of the call below it and the Frequency 95% corresponds to the maximum frequency cutting the 5% of the energy of the call above it (Charif *et al.* 2010). Functions Frequency 5% and Frequency 95% were used to estimate the bandwidth that concentrates 90% (BW 90%) of the energy of the call.

The relative peak power was measured in Raven only for note A in both advertisement and courtship calls. Values were corrected using relative differences in the same recording. I.e., we compared the peak power of the advertisement and courtship calls of the same recordings, subtracting from both measurements the minimum peak power measured. The same was done with three different recordings. Therefore, these values were not absolute, but fit for comparisons between different call types and recordings.

Temporal parameters as note duration, call duration and interval between notes were measured using the function Delta time. We also measure call rate manually, by counting the number of calls in one minute for each record. We did a two sample *t*-test to compare temporal parameters and the relative peak power between advertisement and courtship calls of

B. luctuosa. Recordings were deposited at Fonoteca Neotropical Jacques Vielliard, Unicamp, Campinas, Brazil (FNJV 32378−86).

RESULTS

Courtship and clutches

We observed the courtship of one couple of *Bokermannohyla luctuosa*. The couple was found the dawn of March 9th of 2016, in a pool inside a hallow depression of approximate 20 cm of diameter in the margin of the stream. The observation started at 3:00 am and the courtship finished at 4:15 am. We observed the male calling in this depression for almost 13 minutes before the female approached it. At that point the male was calling oriented to the opposite direction of the female. When the female approached the male, it increased its calling rate from 19 calls/minute to 44 calls/minute. Then, the male (Figure 2A) turned toward the female and approached her. After that, the male placed his hand on female's head for 12 seconds. During that period, the female remained motionless and the male reduced the calling rate from 52 calls/minute (before placing his hand on her head) to 7 calls/minute (just after touching the female). After about five minutes the female moved closer to the male, who jumped onto her back, entering in axillary amplexus. This first amplexus lasted 57 seconds. Three seconds after releasing the female, the male amplected the female again and emitted a short amplectant call (not considered for analysis). After 58 seconds of this second axillary amplexus the male released the female again, remained 12 seconds at her side, and amplected her for the third time, which lasted for 21 seconds. During each of these three consecutive amplexus the female laid eggs. After this, the male pushed the eggs to the extreme of the depression and then amplected the female for a fourth time. This action was repeated three times, lasting 50 seconds and completing six amplexus cycles. After the sixth amplexus, the female hided in the depression's border and male stayed moving the eggs with its hands for about two minutes.

We also observed three clutches in shallow depressions with leaf litter inside them. These depressions were close (5 cm) or far (from 1 to 3 m) from the stream. One observed clutch had eggs of different stages of development (Figure 2B).

Vocalizations

We recorded two distinct vocalizations: the advertisement call, emitted by males while attracting females from long distance to their position, and the courtship call, emitted by males in presence of the female. Their (advertisement) calling activity started on January at 23:00h and lasts during the remaining field period (n = 176 calls). All males were calling inside ground depressions in the border of the stream or in depressions under logs. We recorded nine calling males (FNJV 32377−86), but only four males of those were analyzed (FNJV 32380−81, 32384−85) due to the quality of the recordings.

Both the advertisement and the courtship calls presented pseudo-pulsed structure (Figure 3B) and were are composed by two notes (Figures 3A and C) emitted in different combinations (Table 1). In both calls, note A was longer and had a higher peak of dominant frequency than note B (Table 2). Occasionally, note A when emitted in the advertisement call had a short portion at the beginning of the note separate from a longer second part (Figure 3A).

We analyzed 347 notes of advertisement call from four different males (mean = 86.75 notes/male). Advertisement calls were emitted in a mean rate of 23.5 ± 10.62 calls/minute. This call lasted on average 1.11 ± 0.62 seconds (Table 3), with an average peak of dominant frequency of 537.85 ± 276.09 Hz. We also analyzed 278 notes of courtship calls from three different males (mean = 92.6 notes/male), emitted in a mean rate of 47 ± 9.90 calls/minute. Courtship calls were longer than advertisement calls, emitted with a higher rate and with a lower relative peak power than the advertisement call (Table 3).

DISCUSSION

Acoustic signals and tactile stimuli are an important part of *Bokermannohyla* spp. courtship, in some cases the cues can be harder as in *B. ibitiguara* (where male hit the female with his arm) or a simple touch as in *B. nanuzae* (Nali & Prado, 2012; Lima *et al.* 2014). In this study we saw tactile stimuli similar to that reported for *B. nanuzae, Aplastodiscus leucopygius* and *A. arildae* (all included in the Cophomantinae subfamily *sensu* Duellman *et al.* 2016), in which male touches the female with his hands in different parts of female's body (head and dorsum) (Haddad & Sawaya, 2000; Carvalho Jr. *et al*. 2006, Zina & Haddad, 2007;

Lima *et al.* 2014). Such stimuli are commonly observed in species in which males construct a nest prior to female attraction (Haddad & Sawaya, 2000; Zina & Haddad, 2007). Therefore, it is possible that the depression where the couple was observed could have been previously constructed by the male of *B. luctuosa*.

From our observations we suggest that the advertisement call of *B. luctuosa* was produced to attract the female and the courtship call could stimulate the female to accept males' amplexus and also stimulate its egg laying (Toledo et al 2015). Although the advertisement call structure in the *B. circumdata* group showed to be highly variable, for example, the number of notes varies from one to 30, calls lasts from 0.1 up to 7.6 seconds, and peak of dominant frequency varies from 0.3 to 2.7 kHz (Gaiga *et al.* 2013), we found similarities. In agreement to our results, the advertisement call of species of the *Bokermannohyla circumdata* group is generally composed by pulsed notes as those described for *B. ibitiguara*, *B. nanuzae* and *B. sazimai* (Eterovick & Brandão, 2001). Therefore, we suggest that this character could be shared to all of its

Geographic variations of calls were reported for *B. nanuzae*, *B. circumdata*, and *B. sazimai* (Carvalho *et al.* 2012; Gaiga *et al.* 2013). Advertisement call of *B. luctuosa* was described from the type locality (Serra do Japi, Jundiaí, São Paulo, Brazil: Pombal & Haddad, 1993) and Atibaia (São Paulo, Brazil: Carvalho *et al.* 2012). The advertisement call of population from Atibaia had two different notes emitted consecutively (Carvalho *et al.* 2012), similar to what we described from Botucatu. The mean duration of the call in these three populations (Serra do Japi, Atibaia and Botucatu) is variable (Table S1). The advertisement call of population from Serra do Japi has only one note, and is shorter than the other two populations (Pombal & Haddad, 1993). Note B is longer (0.44 ± 0.046) than note A (0.057 ± 0.02) in individuals from Atibaia (Carvalho *et al.* 2012), contrary to what we observed in Botucatu (note A: 0.77 ± 0.18 s; note B: 0.19 ± 0.05 s). Also, calls of individuals from Atibaia are higher-pitched (0.49 to 2.0 kHz) (Carvalho *et al.* 2012) than calls of the other two populations, the lowest-pitched call is from individuals from Serra do Japi (0.3 to 1.8 kHz) (Pombal & Haddad, 1993). The highest peak of dominant frequency in the population of Botucatu is from note A (1.8 kHz) and in the population from Atibaia is from note B (2.0 kHz). Therefore, we suggest that those notes could be the same as also observed in the spectogram (Carvalho *et al.* 2012) (Table S1).

Courtship call of *B. luctuosa* was never described in previous studies, probably due to the low probability of recording such event in field activities. Many species of the Cophomantinae subfamily emit courtship calls, as *B. nanuzae*, *A. leucopygius*, *A. arildae*, *A.*

perviridis, and *Hypsiboas rosenbergi* (Kluge, 1981; Haddad & Sawaya, 2000; Haddad *et al.* 2005; Carvalho Jr. *et al.* 2006; Lima *et al.* 2014). Some species have more than one courtship call and this call may be variable and it is emitted when the female approaches the male or while guiding the female to the spawning site (Toledo *et al.* 2015).

The courtship call usually presents similar structure to the advertisement call, being the rate of emission and power the main differences. Courtship calls from *H. rosenbergi, A. perviridis* and *B. nanuzae* have a lower rate of emission when compared to their advertisement calls (Kluge, 1981; Haddad *et al.* 2005; Lima *et al.* 2014). On the other hand, *A. leucopygius, A. arildae* (Haddad & Sawaya, 2000; Carvalho Jr. *et al.* 2006) and *B. luctuosa* (present study) have a higher rate of emission in their courtship call compared with its advertisement call. Peak of dominant frequency in courtship calls is usually the same as those of advertisement calls (*B. nanuzae, A. leucopygius, H. rosenbergi*) (Kluge, 1981; Haddad & Sawaya, 2000; Lima *et al.* 2014). However, a lower peak of dominant frequency in the courtship call has been reported for *A. perviridis* (Haddad *et al.* 2005) and presently for *B. luctuosa*. We showed that the relative peak power of the courtship call is lower than the advertisement call, whilst other studies did not analyzed this parameter.

Even few is known about species of the *B. circumdata* group, some of them have been reported to use depressions or burrows near the stream as spawning sites (Nali & Prado, 2012; Lima *et al.* 2014; Centeno *et al.* 2015). However, the use of the same site for different clutches has been reported only for *B. nanuzae* (Lima *et al.* 2014) and *B. luctuosa* (Pombal & Haddad, 1993). Such particular characteristic might indicate that males of these species use the same site to attract different females. More detailed studies about reproductive behavior are needed for species of *Bokermannohyla* from different groups.

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Table 1. Variable composition of the advertisement call of *Bokermannohyla luctuosa* with two notes of unknown function. Values in parenthesis correspond to the percentage of frequency of the call composition in 176 calls.

Calls composition	Frequency in 176 calls
A	92 (52.3 %)
AB	27 (15.3 %)
AABB	13 (7.4 %)
ABB	12 (6.8 %)
AA	8 (4.5 %)
AAB	8 (4.5 %)
AAABB	3 (1.7 %)
AABBB	2 (1.1 %)
BAB	2 (1.1 %)
BBAA	2 (1.1 %)
AAA	1 (0.6 %)
AAABAB	1 (0.6 %)
AABAB	1 (0.6 %)
AABABBB	1 (0.6 %)
AABBAB	1 (0.6 %)
ABAAB	1 (0.6 %)
BB	1 (0.6 %)

Table 2. Acoustic parameters for the notes of the advertisement and courtship calls of males of *Bokermannohyla luctuosa*. Values presented as Mean ± SD (range).

	Note A (N=346)		**Note B (N=232)**	
	Advertisement call	**Courtship call**	**Advertisement call**	**Courtship call**
Note duration (s)	0.73 ± 0.18 (0.19 ─ 1.53)	0.84 ± 0.17 (0.33 ─ 1.48)	0.19 ± 0.05 (0.07 ─ 0.41)	0.19 ± 0.04 (0.08─0.34)
Freq 5% (Hz)	359.55 ± 21.22 (257.80 ─ 539.10)	412.01 ± 96.45 (70.30 ─ 609.40)	304.68 ± 75.12 (46.90 ─ 375.00)	327.95 ± 37.79 (93.80 ─ 468.80)
Peak Freq (Hz)	653.85 ± 433.26 (375.00 ─ 1757.80)	748.65 ± 484.61 (281.20 ─ 1804.70)	376.95 ± 77.44 (70.30 ─ 679.70)	495.11 ± 252.24 (328.10 ─ 1710.90)
Freq 95% (Hz)	1863.85 ± 341.49 (820.30 ─ 2671.90)	2027.18 ± 166.66 (1429.70 ─ 2367.20)	1996.09 ± 656.37 (773.40 ─ 2906.20)	2199.85 ± 425.12 (1148.10 ─ 2976.60)
BW 90% (Hz)	1504.32 ± 337.01 (468.80 ─ 2343.80)	1615.17 ± 197.59 (1078.10 ─ 2015.60)	1691.41 ± 660.21 (445.30 ─ 2695.30)	1871.90 ± 425.59 (820.30 ─ 2718.80)

Table 3. Temporal and spectral parameters for advertisement and courtship calls of *Bokermannohyla luctuosa* and results of individual *t*-tests. Values presented as Mean ± SD (range; sample size). See methods for the explanation of the relative peak power.

	Advertisement call (N = 4 males)	**Courtship call (N = 3 males)**	***t*-test**
Call duration (s)	1.11 ± 0.62 (0.52 – 3.78; 176)	16.24 ± 15.51 (3.87 – 50.49; 10)	t = 13.36; df = 184; P < 0.0001
Intervals between notes (s)	1.72 ± 2.43 (0.01 – 14.91; 313)	0.08 ± 0.03 (0.02 – 0.25; 266)	t = 9.29; df = 577; P < 0.0001
Call rate (calls/minute)	23.5 ± 10.62 (6 – 40; 14)	47 ± 9.90 (36 – 60; 10)	t = 5.49; df = 22; P < 0.0001
Relative peak power (dB)	10.37 ± 3.5 (4 − 16; 30)	2.49 ± 2.80 (0 – 11.2; 30)	t = 9.61; df = 58; P < 0.0001

FIGURES

Figure 1. Study site, indicating the studied forest fragment (A) and the stream inside this fragment (B-C), at the district of Rubião Junior, municipality of Botucatu, state of São Paulo, southern Brazil.

Figure 2. Male of *Bokermannohyla luctuosa* (A), and an egg mass with eggs in different developmental stages (B).

Figure 3. Spectrogram and oscillogram of the advertisement call (FNJV 32381) of *Bokermannohyla luctuosa*, municipality of Botucatu, São Paulo, Brazil (A). Structure of pseudo-pulses in advertisement and courtship calls of *B. luctuosa* (B). Spectrogram and oscillogram of the courtship call (FNJV 32381) of *Bokermannohyla luctuosa*, municipality of Botucatu, São Paulo, Brazil (C).

Figure 1.

Figure 2.

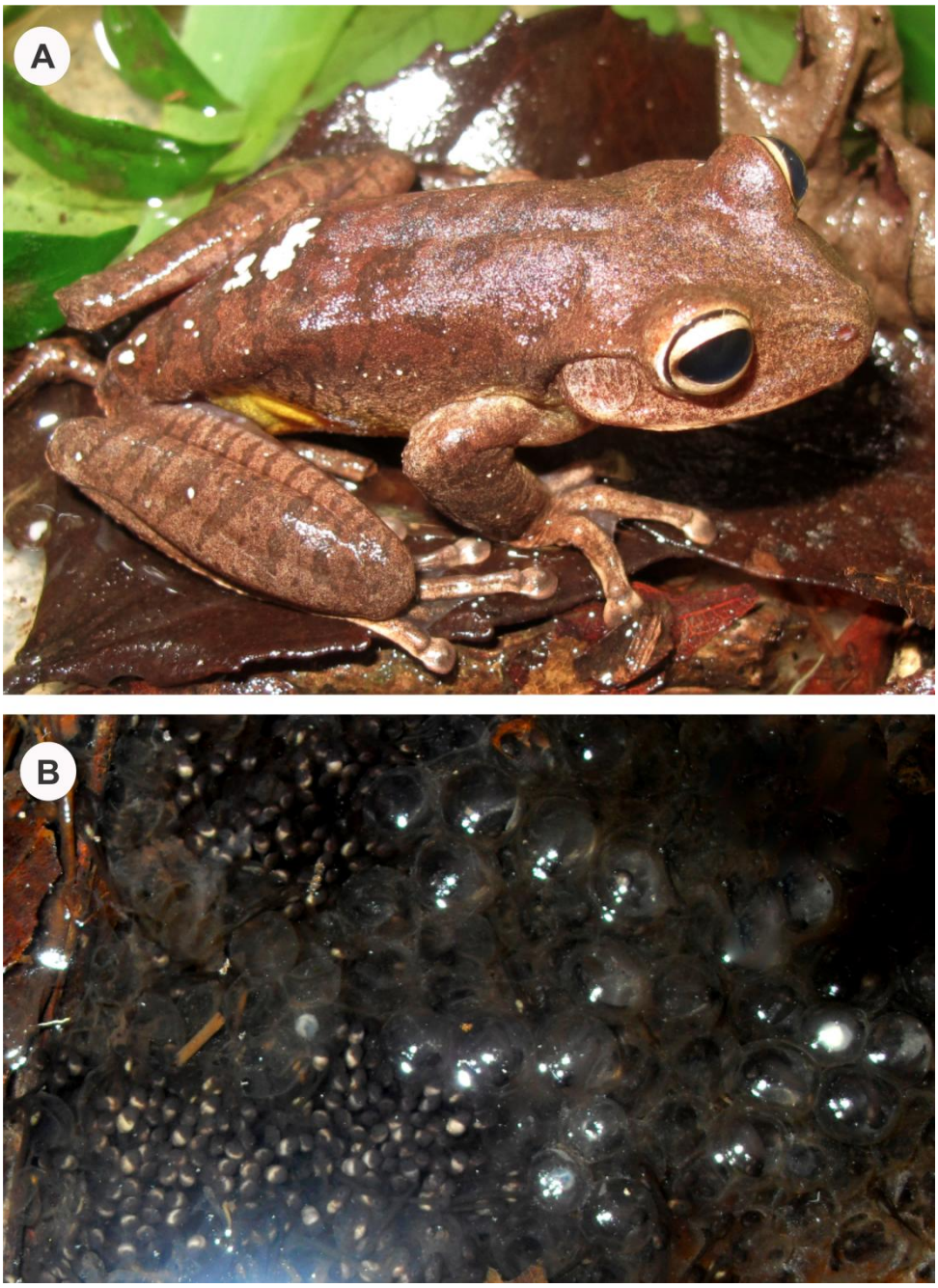
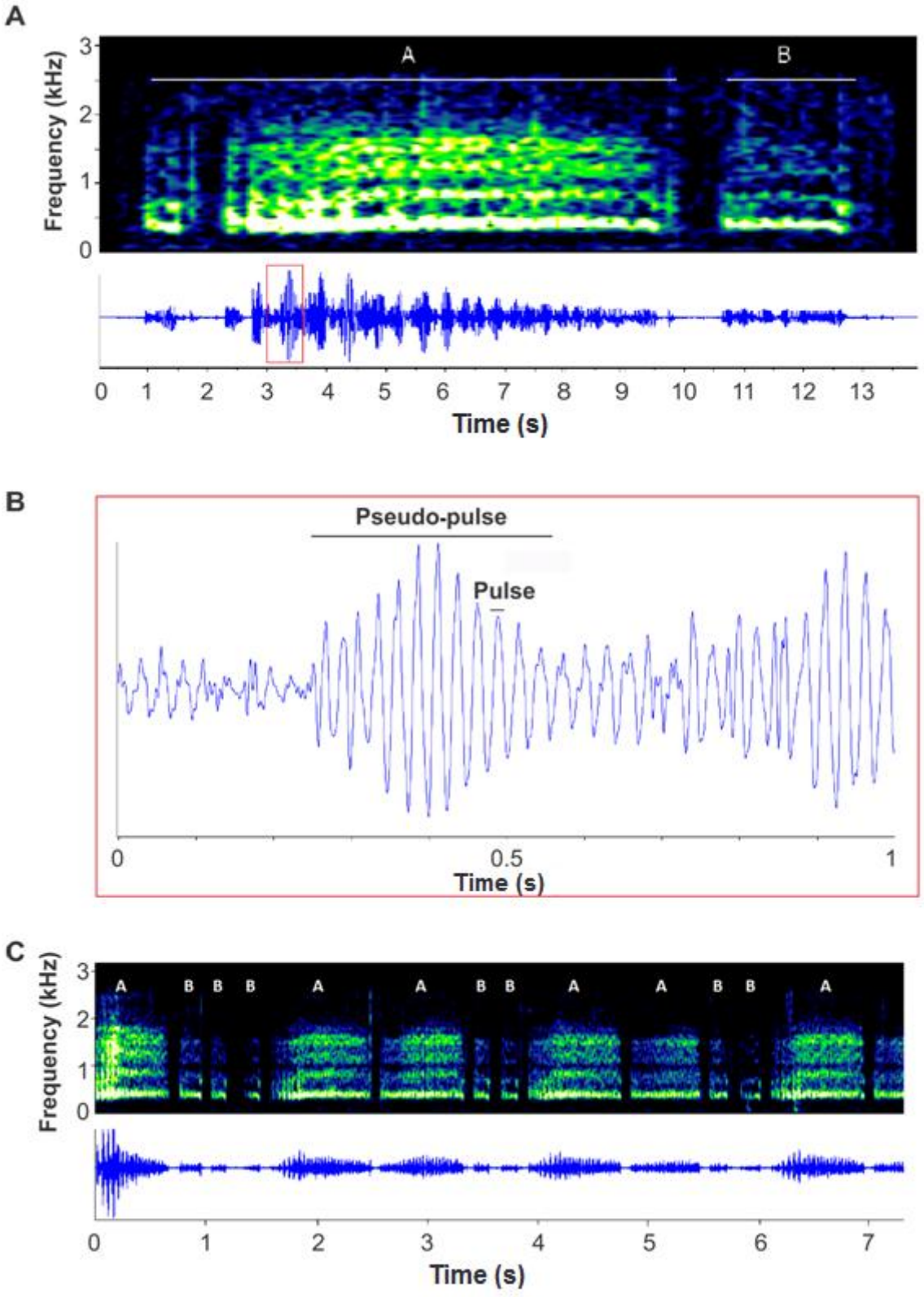


Figure 3.


A
Frequency (kHz)
A
B
Time (s)
B
Pseudo-pulse
Pulse
Time (s)
C
Frequency (kHz)
Time (s)

SUPPLEMENTARY MATERIAL

Table S1. Temporal and spectral parameters for advertisement call of three populations of *Bokermannohyla luctuosa*. Duration values presented as mean ± SD (range), dominant frequency values presented as range.

Population	Note duration (s)		Dominant frequency (Hz)	Publication
	Note A	Note B		
Serra do Japi	0.06		305 ─ 1734	Pombal & Haddad 1993
Atibaia	0.057 ± 0.02	0.44 ± 0.046	490 ─ 2000	Carvalho *et al.* 2012
Botucatu	0.77 ± 0.18 (0.19 ─ 1.53)	0.19 ± 0.05 (0.07 ─ 0.41)	300 ─ 1800	Present study

Considerações finais

Este estudo proporcionou informações sobre as espécies de anuros mudos, encontrando 40 espécies reportadas como mudas, e a relação entre a falta da vocalização de anúncio com o ruído do ambiente. Também encontramos que os resultados da reconstrução do estado ancestral podem variar dependendo o método usado. Assim, detectamos que o estado ancestral ao testar as famílias separadamente foi a presença de canto de anúncio, contrário ao resultado obtido testando as duas famílias na mesma filogenia e inserindo todas as espécies mudas.

Além disso, este estudo forneceu conhecimentos sobre o comportamento reprodutivo de *Bokermannohyla luctuosa,* descrevendo o canto de anúncio e o canto de corte. Também descrevemos o comportamento de corte que envolve sinais acústicos e táteis, similarmente como em outras espécies da mesma subfamília. Com os nossos resultados mostramos que há uma variação geográfica no canto desta espécie, novamente como relatado para outras espécies do gênero.

Baseado nos nossos resultados, destacamos a importância de realizar futuras análises com as outras hipóteses sugeridas para a perda da vocalização de anúncio, assim como adicionar medições de ruído nos habitats das espécies mudas. Embora evidente, também vale ressaltar que a realização de estudos de história natural para as espécies de anuros para as quais ainda não temos informação sobre a presença ou ausência de vocalização é fundamental.

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ANEXOS

Profa. Dra. Rachel Meneguello
Presidente
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"Comunicação acústica em anuros com especial enfoque para: evolução de perda da vocalização de anúncio e presença de vocalização de corte em Bokermannohyla luctuosa"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 08/06/2016

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Comunicação acústica em anuros com especial enfoque para: evolução de perda da vocalização de anúncio e presença de vocalização de corte em Bokermannohyla luctuosa***", desenvolvida no Programa de Pós-Graduação em Ecologia do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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